Reexamination of the Polymerization of Pyridoxylated Hemoglobin with Glutaraldehyde*

M. A. MARINI, G. L. MOORE, S. M. CHRISTENSEN, R. M. FISHMAN, R. G. JESSEE, F. MEDINA, S. M. SNELL, and A. I. ZEGNA

Letterman Army Institute of Research, Division of Blood Research, Presidio of San Francisco, California 94129

SYNOPSIS

Pyridoxylated adult human hemoglobin (HbAo) was prepared using a one molar equivalent of pyridoxal 5-phosphate (PLP) per heme and reduced with either $NaCNBH_3$ or $NaBH_4$. A separate sample was pyridoxylated and passed through a mixed-bed ion exchange column without reduction. All three preparations had a P_{50} of 29 ± 2 torr and a cooperativity of $n = 2.4 \pm 0.1$. These preparations, in both the oxy and deoxy forms, were then treated with 7 equivalents of glutaraldehyde per tetramer at pH 6.8 at 4°C and at room temperature. The polymerization invariably reduced the P_{50} to 18 ± 2 torr with Hill coefficients of less than 2. These solutions, with or without further reduction using $NaCNBH_3$, all retained the PLP in differing amounts (2–3 moles/tetramer). Methemoglobin concentrations were increased during the polymerization reaction. The normal pyridoxylation procedure, using sodium borohydride reduction, resulted in a number of different molecular species. Polymerization with glutaraldehyde caused a further proliferation of molecular species that could not be separated by anion exchange chromatography or by isoelectric focusing. The extent of polymerization, estimated by gel exclusion chromatography and SDS polyacrylamide gel electrophoresis, was from 40 to 50%. Analysis of the reverse phase chromatograms, which separate the heme and the α- and β-chains, showed extensive polymerization and distribution of the radioactively labeled PLP on the protein for all preparations. All of the polymerized and pyridoxylated samples were unstable, and showed different chromatographic patterns after storage at 4°C for 1 month. Attempts to stabilize these preparations by further reduction with $NaCNBH_3$ gave products with a lower P_{50} and lower cooperativity. When the reactions were conducted with a purified HbAo, heterogeneity was somewhat decreased compared to the normally used stroma-free hemoglobin, but a large number of molecular species were still formed.

INTRODUCTION

Use of hemoglobin solutions as plasma expanders offers the desirable properties of low viscosity, oncotic activity, and oxygen and carbon dioxide transport. Extracellular hemoglobin, however, binds oxygen much more tightly than intracellular hemoglobin due, in part, to the loss of 2,3-diphosphoglycerate and to the lower pH of the plasma. This defect may be adequately overcome with pyridoxal 5-phosphate (PLP)[1,2] as well as with a number of other reagents.[3-5] Another disadvantage in the use of hemoglobin solutions for resuscitation therapy lies in the short circulating half-life of native hemoglobin, which is considered to be due to the glomerular filtration of the dimeric form of hemoglobin.[6] To overcome this limitation, various means have been proposed such as encapsulation,[7,8] or chemical modification to form intramolecular and/or intermolecular cross-links. Intratetrameric

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cross-linking with disubstituted aspirin derivatives[9] prevents dimerization, as do the dextran dialdehydes,[10] glycoaldehyde,[5] and 2-nor-2-formylpyridoxal 5-phosphate.[11,12] A common means used to extend the vascular retention of hemoglobin solutions has been by polymerization with glutaraldehyde (GLUT), which forms intra- and intermolecular cross-linked hemoglobin that retards the rate of renal clearance.[1,2,13,14]

The polymerization must be preceded by pyridoxylation to obtain an adequate P_{50} in the end product. Invariably, subsequent treatment with GLUT results in a decrease in the P_{50} and an increase in the concentration of methemoglobin. Increasing the degree of polymerization also decreases the oncotic pressure and increases the viscosity. These are conflicting attributes for a resuscitation fluid since the former property is desirable and the latter is not. Marks et al.[15] have proposed conditions to optimize the balance in these properties during the synthesis of the polymerized product. Most procedures utilize stroma-free hemoglobin (SFH) as the starting material, which results in a complex mixture of all the cellular components of the red blood cell and all of the various derivatives formed by the successive reactions of pyridoxylation, reduction, and polymerization. A study of the reaction products using the procedure of DeVenuto and Zegna,[1] and radioactive PLP, was analyzed by isoelectric focusing and high performance liquid chromatography (HPLC).[16] Isoelectric focusing with radioautography of the intermediate pyridoxylated hemoglobin produced 35 bands, and isoelectric focusing of the polymerized product produced too many bands to separate.[16,17] In a separate report,[18] the reactions of PLP and purified adult human hemoglobin (HbAo) were examined. In this report, the polymerization reaction is studied. Because of the reduced heterogeneity of the pyridoxylated HbAo preparations it was thought to be possible that further information on the nature of the products of the GLUT reaction could be obtained, and that the components could be isolated and characterized.

MATERIALS AND METHODS

HbAo was prepared by the method reported by Christensen et al.[19] The pyridoxylation reactions were as previously reported using a PLP : heme molar ratio of 1 : 1,[18] and the reduced (3 molar equivalents of $NaBH_4$ or $NaCNBH_3$ per heme) and unreduced preparations were treated with GLUT. The ^{14}C-pyridoxyl phosphate had a specific activity of 7.7×10^5 dpm/μmole. GLUT was purchased from Polyscience (Warrington, PA), EM grade, as 50% solutions and from Ted Pella Inc. (Tustin, CA). These were purified by passage through charcoal to remove the material that absorbs at 235 nm.[20] The GLUT was added as a 2.5% solution estimated from the molar absorption of 14.1 at 294 nm.[20] Normally the solutions were freshly prepared from sealed vials stored at −20°C. Occasionally the diluted solutions were stored at −20°C and used within the week.

Polymerization with glutaraldehyde

Aliquots of both the reduced and unreduced pyridoxylated samples were treated with 7 molar equivalents of GLUT per tetramer in both the oxy and deoxy forms at pH 7 (pHstat) at both 4°C and room temperature. Portions of these solutions were then dialyzed overnight at 4°C with three changes of water to remove the excess GLUT. Similar samples were reduced immediately with $NaCNBH_3$ and then dialyzed. All the solutions were then deionized and stored at 4°C for subsequent analysis.

Estimation of Polymerization

The extent of polymerization was estimated by size exclusion on two 0.75 × 30 cm columns of BioSil TSK-250 connected in series, using the Beckman model 322 HPLC. Samples were prepared by dilution of the derivatives in 100 m*M* potassium phosphate, pH 6.9, to a concentration of 0.5 mg/mL. The samples were eluted with the same buffer at 1 mL/min. Samples of ferritin, catalase, aldolase, and bovine serum albumin were eluted at 21.2 min (MW = 440,000), 24.9 min (MW = 232,000), 29.6 min (MW = 158,000), and 31.7 min (MW = 64,000), respectively. Concentrations were estimated from the integration of the chromatograms. Aliquots of the polymerized samples were also applied to 10% cross-linked gels for SDS polyacrylamide gel electrophoresis[21] using the Pharmacia PhastSystem. Approximately 0.5 μg were applied and developed with silver stain.

Radioactivity

Fractions of 0.5 mL were collected from the gel filtration column and each fraction was treated with 0.1 mL of 30% H_2O_2, diluted with 10 mL

Table I Analysis of Pyridoxylated-GLUT Polymerized HbAo

Preparation Polymerized[a]	Reduction[b]	Methemoglobin (%)	P_{50} (torr)	n	PO_4[c] (M/heme)
PLP-HbAo-NR	–	9.1	17	1.4	0.45 ± .08
	+	1.1	11	1.2	0.48 ± .11
PLP-HbAo-BH_4	–	11.0	19	1.9	0.73 ± .09
	+	1.2	18	1.7	0.86 ± .11
PLP-HbAo-$CNBH_3$	–	7.4	18	1.7	0.39 ± .04
	+	1.1	12	1.4	0.31 ± .07

[a] PLP-HbAo-NR: unreduced pyridoxylated hemoglobin; PLP-HbAo-BH_4: pyridoxylated hemoglobin reduced with $NaBH_4$; PLP-HbAo-$CNBH_3$: pyridoxylated hemoglobin reduced with $NaCNBH_4$.

[b] Reduced, after GLUT polymerization, with sodium cyanoborohydride.

[c] Value and standard deviation determined on five samples using the radioactivity.

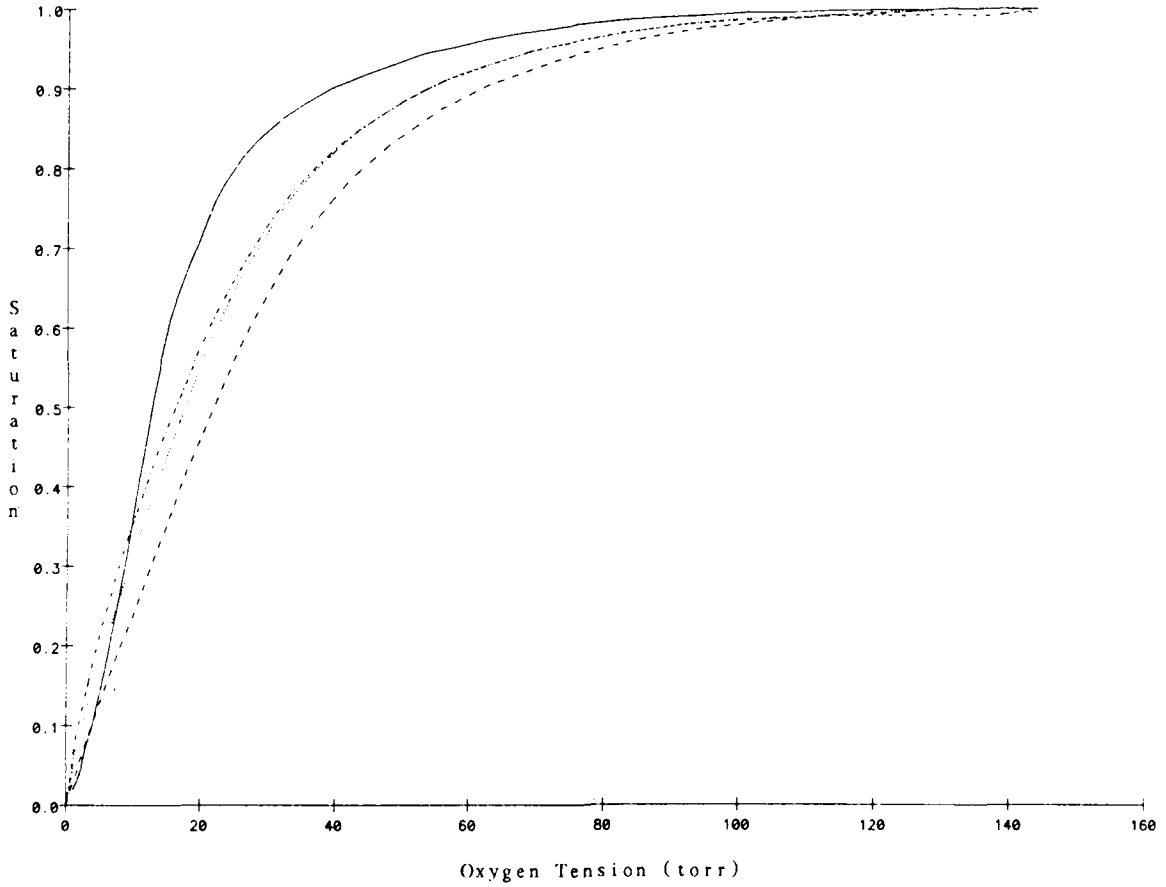

Figure 1. Oxygen equilibrium curves of HbAo (solid line) and the pyridoxylated-polymerized derivatives. The P_{50} values are 17, 18, and 19 torr, respectively, for PLP-HbAo-NR-GLUT (dot-dash), PLP-HbAo-$CNBH_3$-GLUT (dotted line), and PLP-HbAo-BH_4-GLUT (dashed line). The GLUT-polymerized derivatives are not reduced.

OPTI-FLUOR, and counted for 10 min in a Packard Tri-Carb counter. Isoelectric focusing was done with pH 5–8 Phast gels (Pharmacia). An identical set of samples (75 μg) were loaded on LKB PAG-plates (pH 5.5–8.5). Autoradiography of the PAG-plates containing the ^{14}C proteins was done by pressing the air-dried gel to a 8 × 10 inch sheet of Kodak X-omatic-AR x-ray film at room temperature for 2 weeks.

Analytical Procedures

Hemoglobin concentrations were estimated by the cyanomethemoglobin method[22] or by the absorption at 524 nm.[23] The methemoglobin concentrations were estimated by the procedure of Evelyn and Malloy.[24] Oxygen dissociation curves were determined on a Hemox Analyzer (TCS Corp., South Hampton, PA) at 37°C using the Hemox buffer. Values for P_{50} were taken directly from the graphs and the Hill coefficients were calculated from a plot of the logarithmic values of the fractional saturation at 40–75% against the log of the oxygen tension.

Analytical Chromatography

Analytical chromatography of the samples (100 μL, 1 g/dL) was performed on a Pharmacia MonoQ resin column (0.5 × 5 cm) using the a linear gradient Buffer B (0.02*M* Tris HCl, pH 8.0, with 0.4*M* NaCl) into Buffer A (0.02*M* Tris HCl, pH 8.0).[25] Normally 1 mg of the pyridoxylated polymerized reaction mixtures were injected and eluted at room temperature at a flow rate of 1 mL/min. Fractions of 0.5 mL were collected and counted. Reverse-

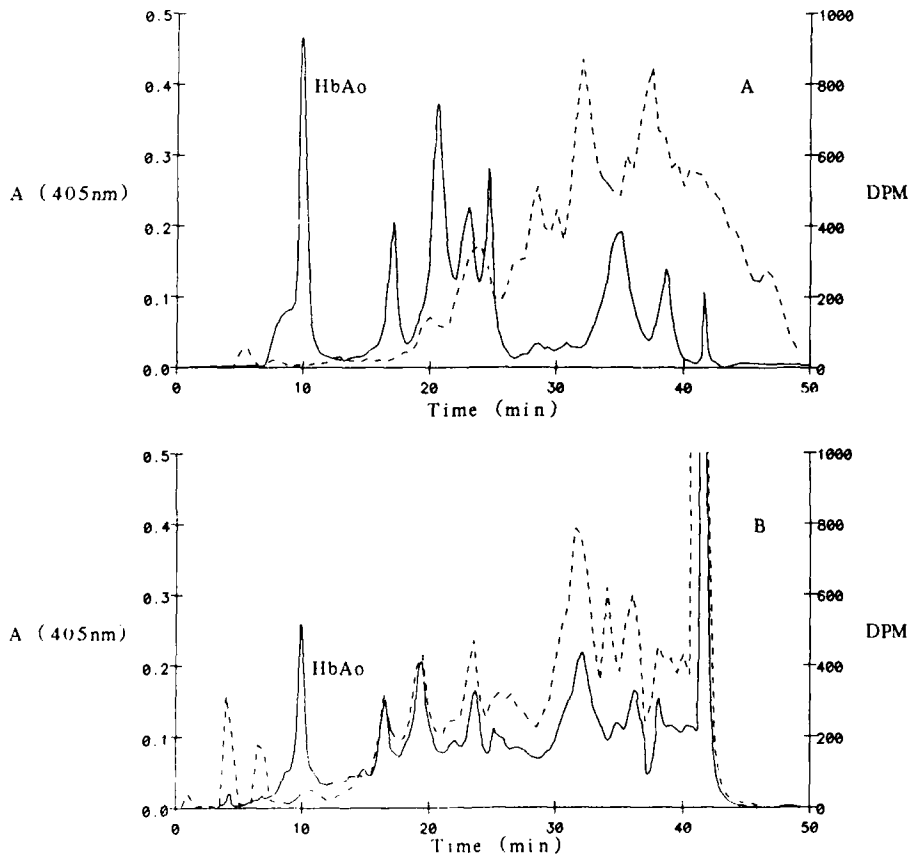

Figure 2. Analytical anion exchange chromatograms of pyridoxylated hemoglobin reduced with sodium borohydride and polymerized with glutaraldehyde (PLP-HbAo-BH_4-GLUT) without reduction. The chromatographic conditions are described in the text. The chromatogram (A) is obtained after dialysis; the separation shown below (B) is that for the same preparation, with the same load, after aging for four weeks at 4°C. The counts are shown as a dashed line.

phase HPLC, by the method of Shelton et al.,[26] was used to evaluate the modification of the globin chains. The separation of the chains was performed on a Waters HPLC with a Wisp injector equipped with a 490 variable-wavelength uv detector and a 820 controller. The column was a Vydac C4 (250 × 4.6 mm) with a 5-μ particle size. A linear gradient of 47% B to 51% B was applied at a flow rate of 1 mL/min for a period of 55 min. After each run, the column was purged with 100% B. Solvent A was aqueous 20% acetonitrile, 0.1% trifluoroacetic acid (TFA) and solvent B was aqueous 60% acetonitrile with 0.1% TFA.

RESULTS AND DISCUSSION

The conditions originally described by DeVenuto and Zegna[1] for the reaction of GLUT with HbAo are quite adequate. This was also the conclusion of Marks et al.[15] and is generally that adopted by others. Temperature of the reaction did not appear to be critical, since similar results are obtained at 4°C as at room temperature. Polymerization of the pyridoxylated derivatives reduced with $NaBH_4$ in the deoxy state (PLP-HbAo-BH_4), gave a product with a slightly higher P_{50} (22 torr vs 19 torr) than that of the oxy derivative with an n value of 2.1 compared to 1.9. Higher concentrations of the GLUT generally caused gel formation and lower concentrations gave lower amounts of polymerization. Hemoglobin concentration also appears to be noncritical and similar results may be obtained with 2–12 g/dL solutions (data not shown). Because the nature of the GLUT cross-links was assumed to be some form of a Schiff base, a final reduction with $NaCNBH_3$ was attempted.

The pyridoxylated species have a P_{50} of 29 ± 2 torr and polymerization with GLUT reduces the P_{50} to 18 ± 2 torr whether reduced or unreduced

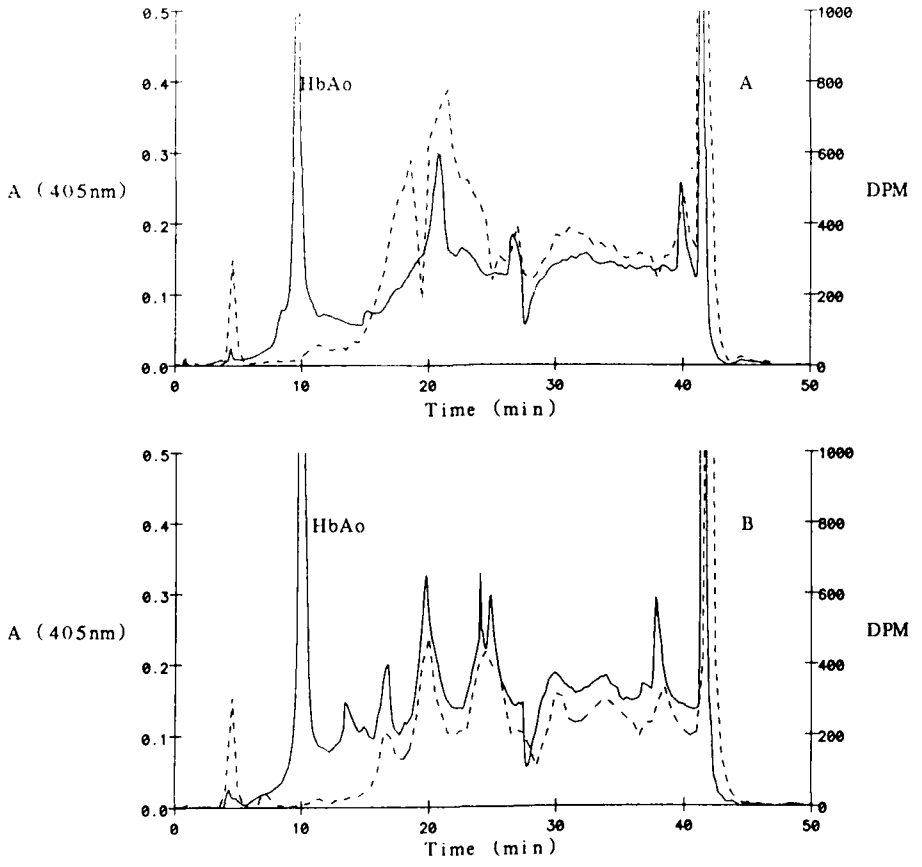

Figure 3. Analytical anion exchange chromatograms of polymerized unreduced PLP-HbAo-NR-GLUT (A) and cyanoborohydride reduced PLP-HbAo-$CNBH_3$-GLUT (B) after 1 month at 4°C. The chromatographic conditions are identical with those of Figure 2. Counts are shown by the dashed lines.

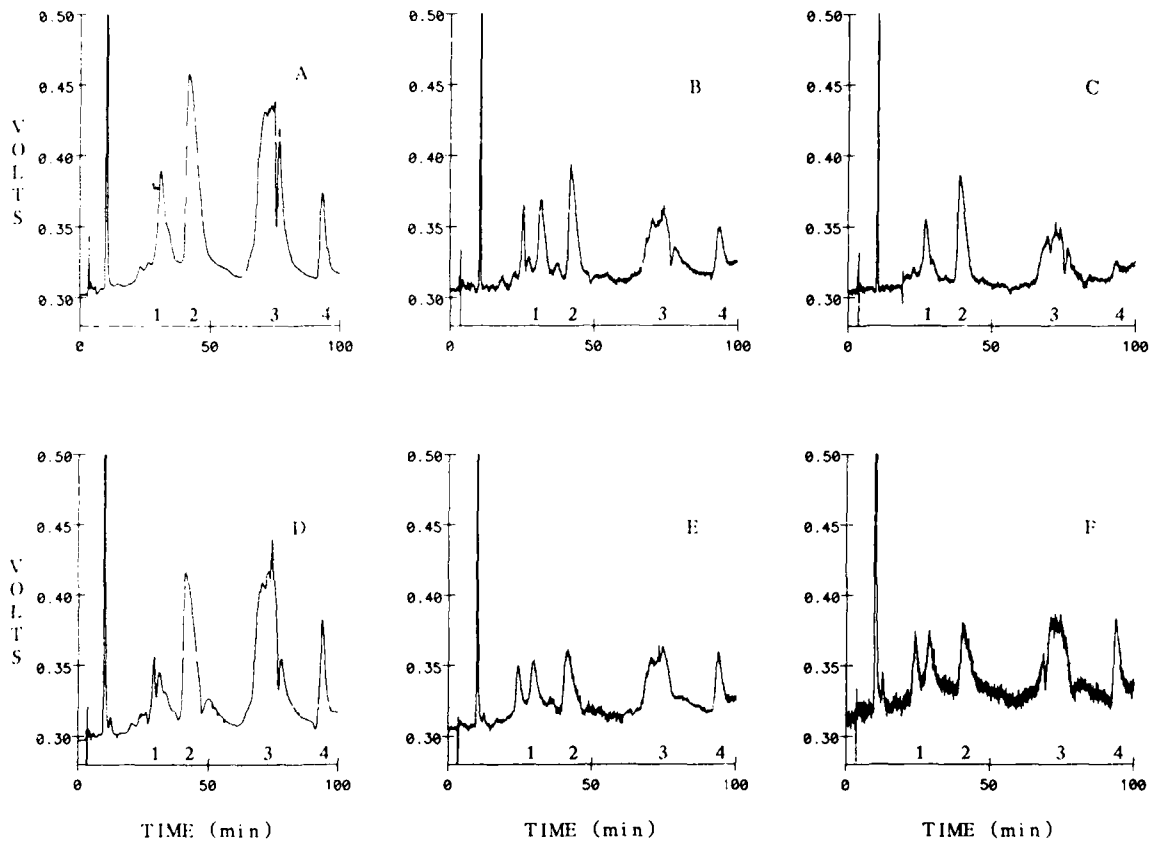

Figure 4. Reverse-phase chromatograms of pyridoxylated-polymerized HbAo preparations. The upper chromatograms are glutaraldehyde treated without a final reduction. (A) PLP-HbAo-NR-GLUT, (B) PLP-HbAo-BH_4-GLUT, and (C) PLP-HbAo-$CNBH_3$-GLUT. The lower series (D–F) are for the identical samples after a final reduction with $NaCNBH_3$. The percentage composition of the peak areas are reported in Tables II and III. The peak at 10 min is free heme. For purposes of comparison, the areas 1 and 2 correspond to the elution times of the β-chains (29 min) and the α-chains (41 min).

pyridoxylated hemoglobin is used (Table 1). The oxygenation curves for the polymerized pyridoxylated samples are shown in Figure 1. As is usually the case, the cooperative nature of the equilibrium was also reduced (Table I) from $n = 2.4 \pm 0.1$ to less than 2. The methemoglobin concentrations are elevated but may be reduced with $NaCNBH_3$. Preparations of the reduced pyridoxylated derivatives retain the PLP (Figures 2b and 3a, b) during the polymerization. The unreduced pyridoxylated hemoglobin polymerized with GLUT (PLP-HbAo-NR-GLUT) also retained the PLP with very little loss, as estimated from the specific activity (Table I).

Analytical chromatography of the reaction products of the pyridoxylated borohydride reduced HbAo polymerized with GLUT (PLP-HbAo-BH_4-GLUT; Figure 2a) indicates that a wide variety of molecular species is present. When this sample is aged for 1 month at 4°C and applied at the same sample load, the chromatogram is altered (Figure 2b), indicating an equilibrium mixture due possibly to the dimer-tetramer equilibrium. It has been reported that an unreducible acetaldehyde adduct of hemoglobin can migrate between peptides.[27] The chromatograms for the unreduced and cyanoborohydride reduced polymerized samples are shown in Figure 3. These also show a different chromatographic pattern after aging. GLUT polymerization produces such a complex mixture of species with regard to both surface charge and molecular weight that the chromatographic techniques used here have not yielded an individual species.

Since GLUT polymerization gave an unstable product (Figure 2), a final reduction was attempted with cyanoborohydride. This causes a further distribution of the label and increases the amount of

Table II Analysis of the Reverse-Phase Fractions of Unreduced GLUT-Polymerized Pyridoxylated HbAo

	PLP-HbAo-NR-GLUT		PLP-HbAo-BH_4-GLUT		PLP-HBAo-$CNBH_3$-GLUT[b]	
Fraction[a]	Area (%)	dpm (%)	Area (%)	dpm (%)	Area (%)	dpm (%)
Peak 1	11.0	22.9	29.0	42.5	16.0	34.3
Peak 2	30.5	16.7	22.1	24.6	36.3	18.7
Peak 3	51.1	56.7	38.8	28.3	43.8	44.9
Peak 4	7.4	4.6	10.1	4.6	3.9	2.1

[a] These fractions are identified in Figure 4. Peaks 1 and 2 correspond to the β- and α-chain regions, respectively.
[b] Polymerization of PLP-HbAo-$CNBH_3$.

Table III Analysis of the Reverse-Phase Fractions of $NaCNBH_3$-Reduced GLUT-Polymerized Pyridoxylated HbAo

	PLP-HbAo-NR-GLUT		PLP-HbAo-BH_4-GLUT		PLP-HbAo-$CNBH_3$-GLUT	
Fraction[a]	Area (%)	dpm (%)	Area (%)	dpm (%)	Area (%)	dpm (%)
Peak 1	11.0	24.2	23.4	41.3	21.7	33.6
Peak 2	30.2	15.9	20.7	19.6	17.2	21.3
Peak 3	48.6	52.9	45.6	31.5	45.1	37.1
Peak 4	10.2	7.0	10.3	7.6	16.0	8.0

[a] These fractions are those identified in Figure 4d–f. All the preparations were finally reduced with $NaCNBH_3$.

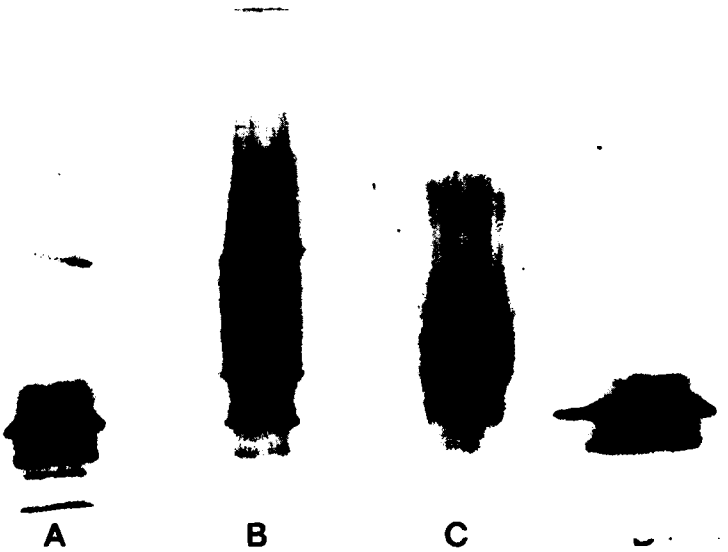

Figure 5. Isoelectric gel electrophoresis (PhastSystem, pH 5–8) of GLUT-treated pyridoxylated derivatives of HbAo and SFH prepared without the final reduction. The gels were developed with silver stain. The samples are (A) SFH, (B) borohydride-reduced pyridoxylated polymerized SFH, (C) borohydride-reduced pyridoxylated polymerized HbAo, and (D) unreacted HbAo. The samples were 0.5 μg.

material in peak 4 (Figure 4d–f). The changes are not extensive and for stability of the preparations, a further reduction may be a desirable additional step, although there is a reduction in the P_{50} as well as in the cooperativity (Table I). The GLUT cross-links could consist of Schiff bases that could conceivably rearrange and/or dissociate. This latter possibility, however, is unlikely since incubation of the unreduced polymerized preparations with glucose-6-phosphate dehydrogenase did not alter the enzymatic activity.

The reverse-phase chromatograms are shown in Figure 4. In all polymerized species, the β-chain is extensively modified with some of the α-chain also modified (Tables II and III). Comparison of the areas of the peak regions (Table II) indicates that the β-chains (peak 1) of the unreduced and $CNBH_3$-reduced preparations are more likely to participate in the polymerization. The most extensively modified β-chains are obtained with the unreduced pyridoxylated HbAo (11% remaining) compared to 29% remaining for the sodium borohydride reduced material. Prior to polymerization, the major fraction of the PLP was found on the β-chains[18] for both the unreduced (PLP-HbAo-NR), and the cyanoborohydride-reduced material (PLP-HbAo-$CNBH_3$). GLUT causes some of the PLP of the unreduced sample and the cyanoborohydride-reduced sample to be found in the α-chain region.

Although the preparations utilizing HbAo are less heterogeneous than similar preparations made with SFH (Figure 5), there were still many molecular species. By isoelectric focusing, the least heterogeneous preparation is the unreduced PLP-HbAo-NR-GLUT but the P_{50} values are low (Table I). The highest P_{50} values are those found with the borohydride-reduced material (PLP-HbAo-BH_4-GLUT), which is the procedure generally used, but these preparations are the most varied and present more difficulty for chemical characterization. The result of SDS-PAGE separation shows a considerable increase in molecular weight (Figure 6) with the PLP distributed throughout the ionic species (Figure 7).

The increase in molecular weight can also be shown using gel filtration chromatography (Figure 8) with the approximate molecular weight distribu-

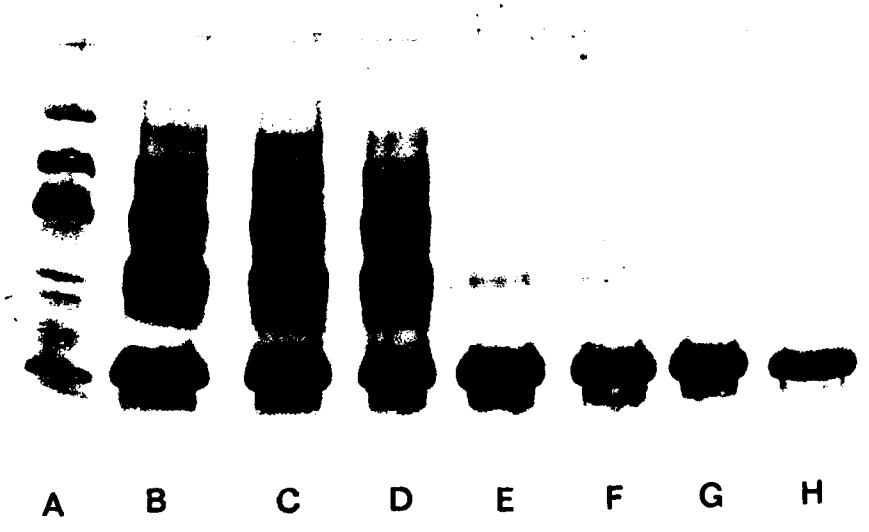

Figure 6. SDS-PAGE electrophoresis of GLUT-treated pyridoxylated derivatives of HbAo on 10–15% cross-linked gels (PhastSystem). Each lane contains 0.5 μg of protein. The samples are (A) standards from 14,300 daltons to 94,000 daltons; (B) PLP-HbAo-BH_4-GLUT; (C) PLP-HbAo-$CNBH_3$-GLUT; (D) PLP-HbAo-NR-GLUT; (E) PLP-HbAo-BH_4; (F) PLP-HbAo-$CNBH_3$; (G) PLP-HbAo-NR; and (H) an unreacted sample of HbAo.

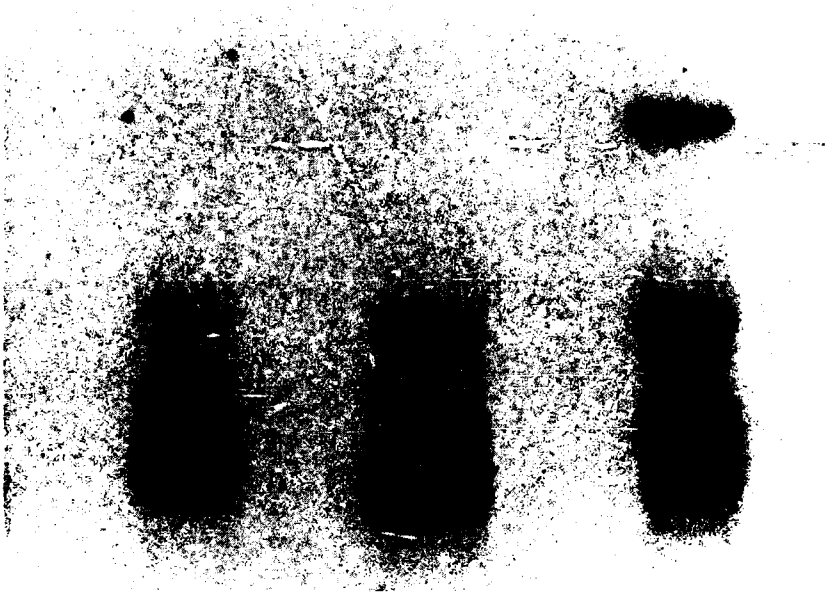

Figure 7. Autoradiography of isoelectric focusing gel (PAGplate, pH 5.5–8.5) of the GLUT-polymerized derivatives. The unreduced preparation (PLP-HbAo-NR-GLUT) is in the left lane. The center lane has the cyanoborohydride-reduced pyridoxylated HbAo (PLP-HbAo-$CNBH_3$-GLUT) and the right lane has the borohydride reduced material (PLP-HbAo-BH_4-GLUT). Each lane contains 75 μg of sample.

tion given in Table IV. This would decrease the oncotic pressure, which is desirable, but it would also increase the viscosity. Polymerization using the unreduced pyridoxylated preparation (PLP-HbAo-NR-GLUT) contains approximately the same amount of ^{14}C PLP (Table I) as the original pyridoxylated product, but a portion of the pyridoxal phosphate is released on gel filtration. After 1 month, however, the amount released is considerably diminished, possibly because of the formation of a stable cyclic imidazolidinone.[27]

Molecular heterogeneity is not necessarily a disadvantage for the functional properties of an emergency resuscitation fluid, but it is a severe liability when the product produces undesirable side effects. It is then very difficult to define the nature or the origin of the deleterious material. SFH is a very complex mixture, and preparations of SFH are not standardized between laboratories and often not within the same laboratory. Further diversity occurs upon reaction with PLP, reduction, and polymerization to produce an undefined mixture of unmodified, modified, and polymerized molecules that will resist chemical characterization (Figures 2, 3, and 5). Moore et al.[17] have calculated that the nonspecific binding of GLUT to a mixture of reduced PLP-SFH-BH_4 species will produce hundreds of products. Still GLUT polymerized pyridoxylated SFH can be used as a blood extender and can successfully transport oxygen.[28-31] Reports that these preparations are antigenic[32] were questioned by Feola et al.[33] Similar preparations are also reported to cause hypoxic and ischemic effects,[34,35] but these may be due to "impurities."[33] SFH causes a significant vasoconstriction,[36,37] but purified HbAo, bis(3,5-dibromosalicyl)fumarate cross-linked SFH,[38] and polymerized pyridoxylated SFH do not.[39]

Because of these conflicting reports, it is well to reexamine the preparative procedures used. It is strongly suggested that the utmost care be exercised in the initial purification of SFH to obtain HbAo in its purest form, and that subsequent reactions be closely monitored not only for yield, but for reproducibility of the physiological and biochemical properties. Even with these precautions, it is not certain that the nature of the derivatives formed with GLUT can be properly

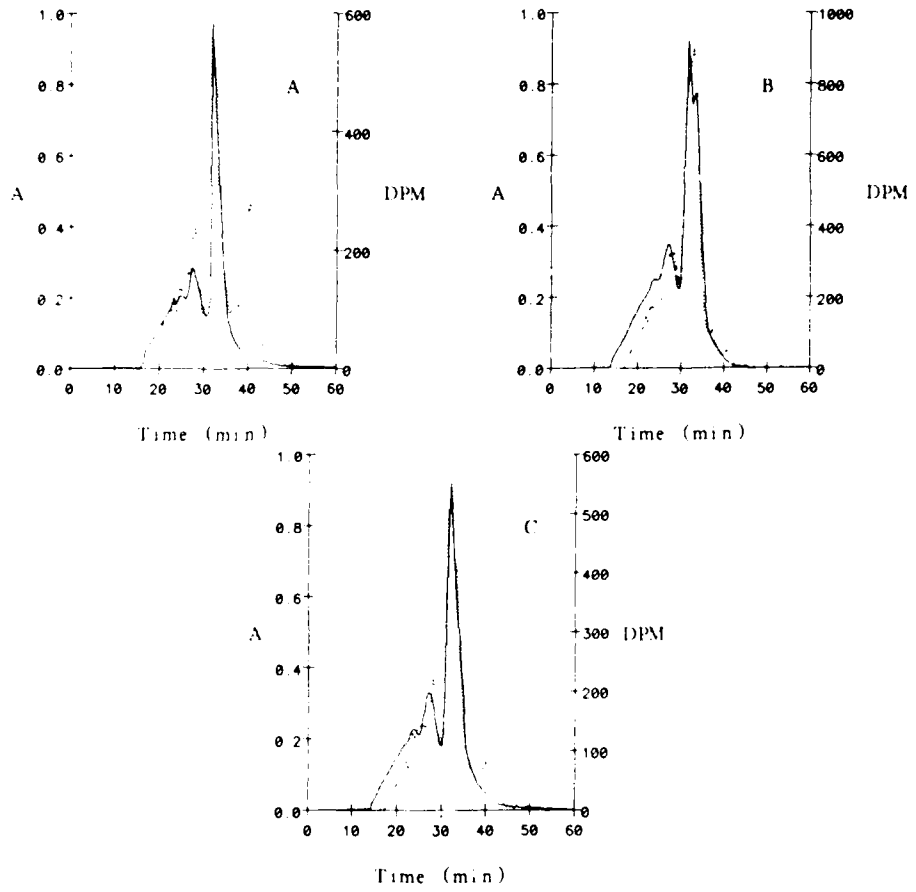

Figure 8. Gel filtration chromatography of polymerized, pyridoxylated HbAo. (A) PLP-HbAo-NR-GLUT, (B) PLP-HbAo-BH_4-GLUT, and (C) PLP-HbAo-$CNBH_3$-GLUT. The counts are shown as the dashed line. The chromatographic conditions are described in the text and the detection is at 280 nm.

Table IV Percentage Composition of Polymerized Pyridoxylated HbAo

Preparation	Molecular Weight[a]			
	64,000	128,000	256,000	> 256,000
PLP-HbAo-NR-GLUT	56.0	21.3	22.3	—
PLP-HbAo-BH_4-GLUT	50.3	18.7	26.0	4.6
PLP-HbAo-$CNBH_3$-GLUT	47.8	18.4	30.9	2.5
PLP-HbAo-NR-GLUT-$CNBH_3$[b]	54.1	19.9	25.4	—
PLP-HbAo-BH_4-GLUT-$CNBH_3$[c]	47.7	18.2	12.3	25.1
PLP-HbAo-$CNBH_3$-GLUT-$CNBH_3$[d]	48.1	19.0	12.2	20.1

[a] Estimated from the elution time of the standards (see text).
[b] Unreduced pyridoxylated HbAo polymerized with GLUT and then reduced with $NaCNBH_3$.
[c] PLP-HbAo-BH_4-GLUT finally reduced with $NaCNBH_3$.
[d] Preparation finally reduced with $NaCNBH_3$.

characterized. This would make it difficult to determine the consistency of the product, which is one of the critical requirements for a blood substitute.

GLUT has long been used as a tissue fixative because of its ability to nonspecifically cross-link proteins and other polymers. The primary reactive sites are the α- and ε-amino groups; however, GLUT will also react with the phenolic ring of tyrosine, the imidazole group of histidine, and the sulfhydryl group of cysteine.[40] The mechanism of the reaction(s) of GLUT with proteins is not understood, and several mechanisms have been proposed with spectral, nmr, and titration data used as partial support for each.[41-43] In aqueous solution, glutaraldehyde exists not as a single species but as a complex equilibrium mixture of many different species.[40,44] GLUT cross-linking at neutral pH could involve an α, β-unsaturated aldehyde to form a Schiff base conjugated to a double bond, which can form variable size cross-links with a variety of bonds.[45,46] The bond(s) are acid stable and would prevent a quantitative estimate of the GLUT incorporated. It must be emphasized that the cross-links formed and aldehyde incorporated are both pH and concentration dependent,[41] and that the product is not stable (Figure 2a, b).

When all the theoretical difficulties and all the problems associated with the standardized preparation of an emergency resuscitation fluid are considered, it is remarkable that many of the proposed candidates are both functional and minimally deleterious. Rigorous adherence to the purification of the starting materials and to the standardization of the preparative procedures should eliminate a number of the anomalous effects.

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